TECHNICAL MANUSCRIPT 65

SOME INTERMEDIATE INFECTIOUS FORMS OF EASTERN AND VENEZUELAN EQUINE ENCEPHALITIS VIRUSES

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ABSTRACT

Several infectious entities were produced by treatment of purified EEE and VEE viruses with deoxycholate, digitonin, and acid (pH 4.0). These artificially produced incomplete forms were compared with the naturally occurring incomplete forms. The relationships of these units to each other, to free infectious RNA (obtained by phenol extraction), and to the intact virus are discussed.

SOME INTERMEDIATE INFECTIOUS FORMS OF EASTERN (EEE) AND VENEZUELAN (VEE) EQUINE ENCEPHALITIS VIRUSES

As described previously by Wecker,[1,2] cells infected with western equine encephalomyelitis (WEE) virus or eastern equine encephalomyelitis (EEE) virus contain, in addition to the mature virus particle, a viral component containing the full complement of the ribonucleic acid (RNA) and here termed immature virus. We have found that this immature virus is also present in cells infected with Venezuelan equine encephalomyelitis (VEE) virus.

In tissue culture supernatant fluid of chick fibroblast monolayers infected with EEE or VEE viruses there is a ribonuclease-sensitive source of infectious RNA (IRNA) that is extractable with cold phenol and that has characteristics similar to the precursor present inside infected cells. This report compares the incomplete virus, which we think is precursor, found in tissue culture supernate with infectious entities obtained by various treatments of purified mature virus.

Chick embryo monolayers grown in lactalbumin hydrolyzate medium containing ten per cent calf serum were used throughout this study. The virus assays were performed by a plaque method employing chick embryo fibroblast monolayers in 60-mm plastic Petri dishes seeded with an initial cell concentration of 20×10^6 cells per plate.[3] Cultures were grown at 37°C in a humidified incubator containing 95 per cent air and 5 per cent carbon dioxide. The virus preparation was diluted in beef heart infusion broth, hereafter referred to as isotonic diluent. Before inoculation with virus, the monolayers were washed with Saline A containing salts in isotonic concentrations. Each plate was inoculated with 0.1 milliliter of diluted virus suspension. After an adsorption period of 20 minutes the inoculum was removed and each plate received five milliliters of agar overlay consisting of Hanks' BSS, 1.1 per cent agar, 0.1 per cent yeast extract, 0.5 per cent gelatin, 0.5 per cent lactalbumin hydrolyzate, 0.14 per cent sodium bicarbonate, and 500 micrograms of DEAE-dextran. After 48 hours the cultures were stained with neutral red and the plaques were counted. This method is normally used to assay intact virus and will be referred to as the isotonic or virus method. Infectious RNA was assayed in monolayers similarly prepared, but the infectious RNA was diluted in a hypertonic solution containing 1M NaCl-0.1M Tris·HCl, pH 8.2. Before inoculation with the RNA, the monolayers were washed with 0.5M NaCl and with the hypertonic diluent. This method is normally used to assay IRNA and will be referred to as the hypertonic or RNA method.

The immature virus that is present inside the cells as described by Wecker[2] is characterized by two properties that distinguish it from mature virus: (a) infectious RNA can be extracted from immature virus by phenol at low temperatures, but hot phenol is required to extract infectious RNA from intact forms of the viruses under study; and (b) its infectious RNA

is not completely protected against ribonuclease. Table I shows that the tissue culture supernate from infected cell cultures contained, in addition to mature virus, an entity that yielded infectious RNA upon extraction with cold phenol and that was susceptible to added ribonuclease. This extracellular entity therefore had the same characteristics, in these two respects, as the intracellular immature virus. In addition, the fact that this infectious form can be recovered from the serum-containing medium suggests that, unlike free RNA, it is at least partially protected against ribonuclease. Phenol-extracted infectious RNA preparations showed no infectivity after incubation in the lact-calf growth medium for less than one minute at 37°C.

Figure 1 shows a comparison of the stabilities of intact virus and IRNA in McIlvaine's and borate buffers over the pH range of 2.0 to 10.0 at 50°C. For infectivity assay, the intact virus was diluted in the isotonic diluent and the infectious RNA in the hypertonic diluent. The infectivity of the RNA was stable over a wide pH range from 4 to 9; on the other hand, the intact virus was stable only from pH 7.3 to 9.3. It was assumed that the incomplete forms of VEE and EEE viruses more closely resembled free RNA than complete virus and would behave like RNA upon acidification at pH 4. If this assumption were true, a very convenient way would be provided for eliminating the infectivity of the intact virus, and we should then be able to demonstrate the infectivity of the incomplete form. We could not use the treatment of virus at pH 4 as planned because, as seen in Table II, acidification of mature virus at pH 4 releases infectious RNA, or an entity that behaves like it. Virus from infected chick embryos was purified by trypsin and ribonuclease treatments and was washed three times to eliminate the enzymes. Thus this purified preparation contained no detectable incomplete virus. The preparation was titrated in both systems, acidified at pH 4, and again titrated in both systems. This preparation was finally treated with ribonuclease after acidification and again assayed by both methods. The purified virus formed plaques more efficiently in the isotonic system, as expected. The virus after acidification formed plaques more efficiently in the hypertonic system. Since no incomplete virus was present, it was concluded that infectious RNA was released from the intact virus by the acidification. The final observation in this experiment was that after acidification, the infectivity exhibited by both methods was eliminated by treatment with ribonuclease. From these results and the fact that infectious RNA is quite stable at pH 4, it appeared that acidification of intact virus at pH 4 released infectious RNA or an entity with infectivity resembling that of the RNA prepared by phenol extraction.

The following experiment was designed to demonstrate that the incomplete virus, as it exists in tissue culture supernate, does not require acidification to exhibit infectivity in the hypertonic system (Table III). Tissue culture supernatant fluid was centrifuged for two hours at 78,000g. A volume of ten milliliters was taken from the upper level of the supernate and centrifuged at 78,000g for two more hours. The supernate from the second centrifugation was assayed by both methods before and after ribonuclease treatment. The data clearly demonstrate that there is present, in

TABLE I. PRESENCE OF IMMATURE VIRUS IN TISSUE CULTURE SUPERNATE

PREPARATION	INFECTIVITY, pfu per ml	
	Virus	RNA[a]
Infected T.C. Fluid (EEE virus)	$10^{9.0}$	$10^{8.4}$
Infected T.C. Fluid (EEE) plus Ribonuclease[b]	$10^{9.1}$	$<10^{2.0}$
Infected T.C. Fluid (VEE virus)	$10^{8.4}$	$10^{5.3}$
Infected T.C. Fluid (VEE) plus Ribonuclease[b]	$10^{8.4}$	$<10^{2.0}$

a. Cold phenol extraction.
b. Fifteen minutes at 37°C, 50 micrograms per ml.

TABLE II. EFFECT OF ACIDIFICATION ON PURIFIED EEE VIRUS

PREPARATION	ISOTONIC TITER, pfu per ml	HYPERTONIC TITER, pfu per ml
Purified EEE virus[a]	$10^{9.4}$	$10^{8.1}$
Purified EEE virus at pH 4[b]	$10^{3.8}$	$10^{4.3}$
Purified EEE virus at pH 4 plus Ribonuclease[c]	$<10^{1.7}$	$<10^{1.7}$

a. Purified by treatment with trypsin and ribonuclease.
b. Purified virus was incubated at pH 4 for 1 hour at 25°C.
c. The purified virus after acidification was incubated with the enzyme at 37°C for 30 minutes.

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Infected T.C. Fluid (VEE virus)	$10^{8.4}$	$10^{6.3}$
Infected T.C. Fluid (VEE) plus Ribonuclease[b]	$10^{8.4}$	$<10^{2.0}$

a. Cold phenol extraction.
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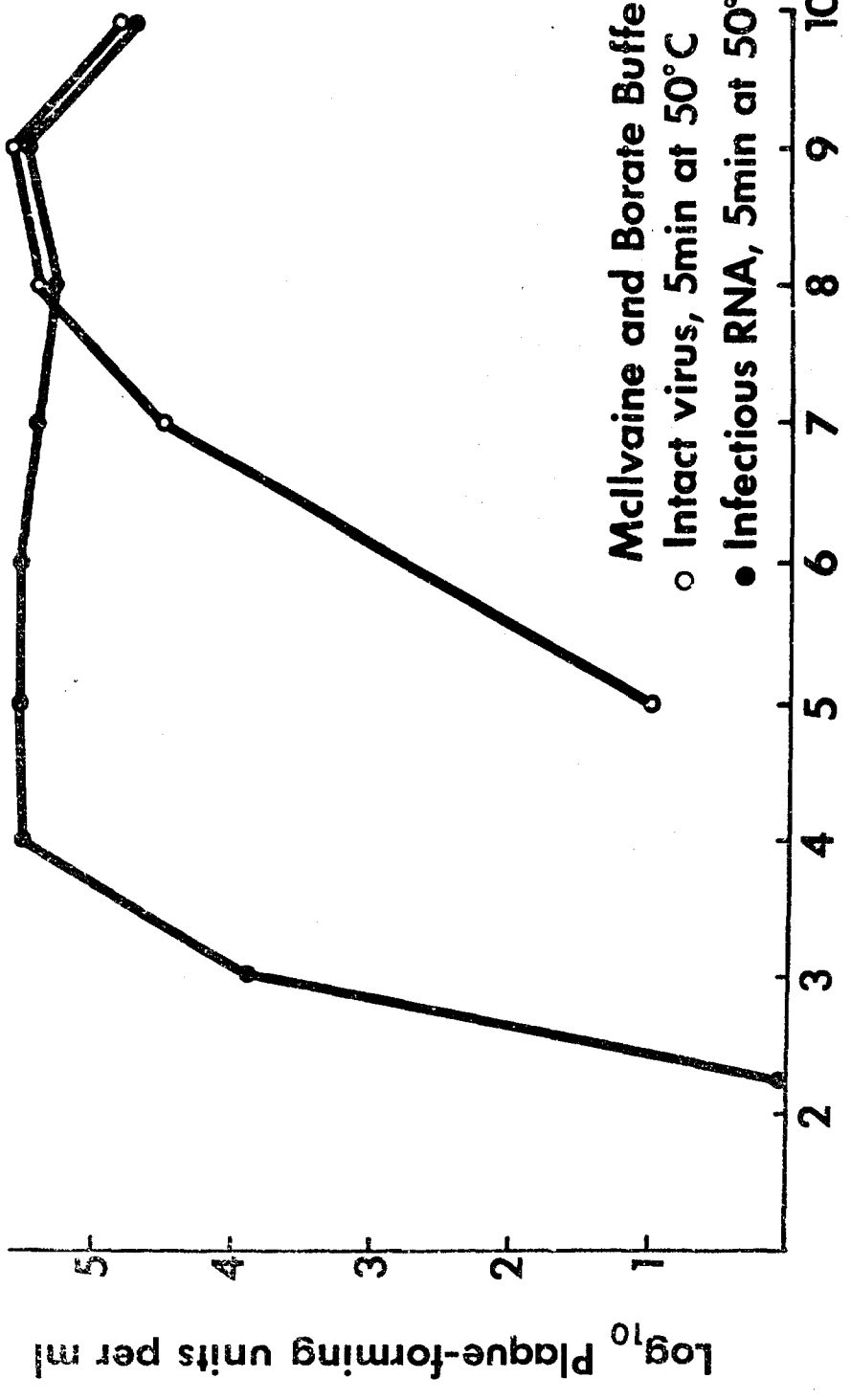

Figure 1. Effect of pH on Intact Virus and Infectious RNA.

the supernate, a ribonuclease-sensitive principle that forms plaques by the hypertonic method. Since there is still mature virus present in the supernate, it is difficult to demonstrate whether or not this incomplete virus has infectivity in the isotonic assay method. We will attempt a separation of the two particle types by density gradient centrifugation and hope that we may then examine the infectivity of the incomplete virus more critically.

TABLE III. EFFECT OF RIBONUCLEASE ON TITERS IN THE SUPERNATE FROM TISSUE CULTURES INFECTED WITH EEE VIRUS

TREATMENT OF SUPERNATE	ISOTONIC SYSTEM, pfu per ml[a]	HYPERTONIC SYSTEM, pfu per ml
None	$10^{8.8}$	$10^{5.4}$
50 μg Ribonuclease[b]	$10^{8.2}$	$10^{4.5}$
Cold Phenol Extraction	$<10^{1.0}$	$10^{3.9}$
50 μg Ribonuclease plus Cold Phenol Extraction	$<10^{1.0}$	$<10^{1.0}$

a. Pfu = plaque-forming units in chick fibroblast monolayers.
b. Supernate and enzyme were incubated at 37°C for 30 minutes.

In 1959 Anderson and Ada[4] reported the recovery of a ribonuclease-sensitive infectious principle after treatment of crude Murray Valley encephalitis virus with sodium deoxycholate. We have studied the effect of deoxycholate on purified EEE and VEE viruses (Table IV). A purified virus preparation with a titer of 10^{10} plaque-forming units (pfu) per milliliter was diluted hundredfold in phosphate buffer, pH 7.4. Deoxycholate was added to final concentrations of 0.5 per cent, 0.25 per cent, and 0.125 per cent and mixed for one minute at 25°C. The treated virus was assayed by both methods before and after ribonuclease treatment. The yield of ribonuclease-sensitive infectious particles released by the deoxycholate treatment, as demonstrated by infectivity in the hypertonic method, was 0.1 per cent of the original concentration of virus. Notice that titers shown in the isotonic method decrease with increasing deoxycholate. Titers in both methods were reduced by treatment with ribonuclease. Our results correspond with those of Richter and Wecker[5] in their study of the effect of deoxycholate on EEE virus.

TABLE IV. EFFECT OF DEOXYCHOLATE AND DIGITONIN ON EEE VIRUS

TREATMENT OF PURIFIED EEE VIRUS	ISOTONIC SYSTEM, pfu per ml	HYPERTONIC SYSTEM, pfu per ml
None	$10^{7.7}$	$10^{5.7}$
Ribonuclease[a]	$10^{7.7}$	$10^{5.7}$
Deoxycholate		
0.5%	$<10^{1.7}$	$10^{4.4}$
0.25%	$<10^{1.7}$	$10^{4.3}$
0.125%	$10^{3.1}$	$10^{4.4}$
0.5% plus Ribonuclease	$<10^{1.7}$	$<10^{1.7}$
Digitonin		
1.0%	$10^{4.6}$	$10^{4.7}$
0.5%	$10^{4.6}$	$10^{4.4}$
0.25%	$10^{4.6}$	$10^{4.6}$
0.25% plus Ribonuclease	$10^{4.4}$[b]	$<10^{1.7}$

a. Ribonuclease treatment = 20 μg per ml ribonuclease for three minutes at 37°C.

b. This titer was reduced when particles, diluted in isotonic diluent, were assayed on monolayers washed with hypertonic diluent.

The effect of digitonin, which is a saponin known to precipitate cholesterols, was also tested with EEE virus. These results are also shown on this slide. Digitonin was added to the purified virus at the indicated concentrations and mixed for 1.5 minutes at 25°C. Both starting and treated materials were assayed by both systems. In the case of the purified virus the titer in the hypertonic system was one per cent of that in the isotonic system; the treated virus showed the same titer by both methods. When the digitonin-treated virus was treated with ribonuclease, the titer by the hypertonic method was reduced and that by the isotonic method was not. The titer in the isotonic system was not due to complete virus in this instance. This was shown by a reduction in titer when the digitonin-treated virus, after ribonuclease treatment, was titered in two additional systems: (a) when it was diluted in isotonic diluent but inoculated onto monolayers that had been washed with hypertonic diluent; and (b) conversely, when it was diluted in hypertonic diluent and assayed on monolayers washed only with isotonic salt solution. These characteristics definitely showed that the particle was not free RNA or mature virus, but rather suggest an intermediate form, probably a ribonucleoprotein.

A protein[6] obtained from partially purified EEE virus (from chick embryo) was mixed with infectious RNA that was prepared by cold phenol extraction. The mixture was incubated for one hour at room temperature and then titrated by both methods. Table V shows the results of a typical experiment. The incubation mixture containing virus RNA and protein showed more plaques in the isotonic system than did the infectious RNA by itself. In several other experiments in which infectious RNA was similarly incubated with non-viral protein, no increase in titer in the virus assay system was observed. This might indicate that some of the viral protein combined with the RNA and thus protected it or made it more infectious when measured by the isotonic method.

TABLE V. EFFECT OF MIXING VIRAL RNA WITH VIRAL PROTEIN[a]/

INCUBATION MIXTURE	ISOTONIC SYSTEM, pfu per ml	HYPERTONIC SYSTEM, pfu per ml
Infectious RNA	$10^{1.7}$	$10^{0.8}$
Infectious RNA plus Viral Protein	$10^{2.8}$	$10^{0.7}$
Viral Protein	$<10^{0.7}$	$<10^{0.7}$

a. Protein incubated with infectious RNA at room temperature for one hour.

Table VI is a summary of the characteristics of the various entities studied here. From the fact that all forms except intact virus were susceptible to ribonuclease and all forms except intact virus yielded infectious RNA by cold phenol extraction, one can conclude that they are incomplete virus forms. In the future these particles will be compared by density gradient centrifugation in sucrose.

Work of others with EEE and WEE viruses has demonstrated the occurrence of an intracellular immature form of virus, the RNA of which is susceptible to the action of ribonuclease. Wecker and Richter[7] have recently succeeded in separating the immature and mature particles by sucrose gradient centrifugation.

TABLE VI. INFECTIOUS PRINCIPLES OF EEE AND VEE VIRUSES AND THEIR PROPERTIES

PROPERTY	MATURE VIRUS	INCOMPLETE VIRUS[a]	DIGITONIN PRINCIPLE	DEOXYCHOLATE PRINCIPLE	pH 4 PRINCIPLE	PHENOL RNA
RNA Extractive by Cold Phenol	NO	YES	YES	YES	YES	YES
Ribonuclease Sensitivity	NO	YES	YES	YES	YES	YES
Infectivity,[b] Isotonic Method	100	?	100	<0.2	1	0.01
Infectivity,[b] Hypertonic Method	1-10	+	100	100	100	100

a. From supernate of infected chick embryo fibroblast monolayers.
b. 100 represents the value of the titer for each particle in the system in which it shows the greater titer; other value shows the relative titer n the second assay system.

In our present work, incomplete virus forms were produced artificially from purified virus in which we have effectively eliminated the immature forms by extensive ribonuclease treatment. Therefore, the source of the infectious principles released by deoxycholate, digitonin, and acidification must be the mature virus. The principles formed by acidification and by vigorous treatment with deoxycholate had characteristics similar to those of the infectious RNA prepared by phenol extraction. The incomplete forms resulting from treatment with digitonin or from mild treatment with deoxycholate exhibited characteristics different from free RNA prepared by phenol extraction. Observations by electron microscopy show the intact virus to consist of a spherical core, about 30 millimicrons in diameter, enclosed in an envelope. On the basis of the chemical composition of the virus, the core probably is nucleoprotein; the envelope or envelopes are more likely lipoprotein in nature.[8] Although these incomplete particles that were produced artificially may not be similar to the natural ones, it is interesting that they can be obtained by chemical treatments. Such an approach might shed some light on how the envelope of the mature virus develops.

It is also expected that the reactions with the chemical agents and methods described will be useful in analyzing the chemical and antigenic components of intact virus particles.

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